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L-Arginine prevents metabolic effects of high glucose in diabetic mice

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Abstract **We tested the hypothesis that activation of the polyol pathway and protein kinase C (PKC) during diabetes is due to loss of NO. Our results show that after 4 weeks of streptozotocin-induced diabetes, treatment with L-arginine restored NO levels and prevented tissue accumulation of sorbitol in mice, which was accompanied by an increase in glutathiolation of aldose reductase. L-Arginine treatment decreased superoxide generation in the aorta, total PKC activity and PKC-β_{II} phosphorylation in the heart, and the plasma levels of triglycerides and soluble ICAM. These data suggest that increasing NO bioavailability by L-arginine corrects the major biochemical abnormalities of diabetes.**

Keywords: Nitric oxide; Diabetes mellitus; Cell adhesion molecule; Signal transduction; Inflammation

1. Introduction

Chronic metabolic changes caused by diabetes establish an inflammatory state, accelerate atherogenesis, and increase the risk of cardiovascular fatality [1,2]. Cellular studies show that exposure to high glucose induces several metabolic abnormalities that include an increase in the activity of the polyol pathway, activation of protein kinase C (PKC), and the generation of reactive oxygen species (ROS) [3]. Of these, activation of the polyol pathway is a key metabolic alteration, which appears to be upstream of PKC and ROS, because inhibition of aldose reductase (AR), an enzyme that catalyzes the first and the rate-limiting step of the polyol pathway, prevents PKC activation and ROS generation [4]. Another major biochemical alteration during diabetes is a decrease in NO bioavailability. Total vascular production of NO is decreased in diabetes [1,2] and gene transfer of endothelial NOS [5] or overexpression of GTP cyclohydrolase I [6], which generates the NOS cofactor, tetrahydrobiopterin, diminishes type 1 diabetes (T1D)-induced endothelial dysfunction. Nevertheless, the relationship between NO and the metabolic abnormalities due to AR, PKC, and ROS remains unclear. In vitro, nitrosoglutathione inhibits AR by inducing S-glutathiolation of the protein [4]. Thus, NO could potentially regulate biochemical pathways of diabetic injury by inhibiting AR. The in vivo validity of this regulatory axis, however, has not been tested.

We hypothesized that in diabetic animals, increasing NO production by L-arginine treatment would inhibit AR and the downstream events leading to PKC activation and ROS production. Treatment with L-arginine has been shown before to regulate hyperglycemia and dyslipidemia [7] and inhibit the polyol pathway [8] in diabetic rats. To examine the effects of L-arginine, we used a streptozotocin (STZ) model of diabetes to examine how an increase in NO bioavailability would affect the major biochemical defects induced by high glucose. Our results show that treatment with L-arginine increased S-glutathiolation of AR in diabetic animals and that this was associated with a decrease in sorbitol accumulation, PKC-β activation, and ROS generation. These findings reveal a novel regulatory pathway in which NO acts as a negative regulator of metabolic abnormalities induced by diabetes.

2. Materials and methods

2.1. Animal studies

Male C57BL/6 mice were obtained from Jackson Labs at 6 weeks of age. Mice were injected (i.p.) with a single dose of approximately 165 mg/kg STZ or an equal volume of 0.05 mol/l citrate buffer, pH 4.5 [6]. Blood glucose was measured 3 days after injection using a HemoCue blood glucose analyzer. Only animals with a blood glucose >400 mg/dl were included in the study. No animals died during the procedure. After 2 weeks of hyperglycemia, mice were implanted with subcutaneous osmotic pumps under anesthesia induced by 300 mg/kg avertin. The pumps delivered either sterile saline or L-arginine at a dose of 50 mg/kg/day. The mice were fed a normal chow diet for an additional 2 weeks. All animals were treated according to institutional guidelines.

2.2. Measurement of NO_x, plasma lipids, sICAM, and TNF-α

Plasma levels of NO_x were measured using the Greiss method. Plasma triglycerides were measured using Wako L Type TG H ELISA kits. Lipoprotein particle size was measured by NMR analysis (LIPOSCIENCE, Raleigh, NC). Mouse sICAM was measured by ELISA (Amersham). Plasma TNF-α was measured using the Mouse TNF-α Ready-SET-go ELISA kit (eBioscience Inc.).

2.3. Measurement of superoxide generation

Superoxide production in sections of mouse aorta was detected using dihydroethidium (DHE) as previously described [9]. Fluorescent images were acquired with a Zeiss LSM 500 microscope and fluorescent intensity was quantified using the MetaMorph software (Universal Imaging).

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2.4. Determination of PKC activity and PKC-β_{II} phosphorylation

Membrane-bound PKC activity was measured using the Promega SignaTECT PKC assay system. The extent of PKC-β_{II} phosphorylation was measured by Western blot using antibodies that specifically recognize phosphorylation of threonine 641 (AbCam). For normalization, the blot was stripped and reprobed with antibodies that recognize total PKC-β_{II} (Santa Cruz Biotechnology).

2.5. Measurement of sorbitol and AR glutathiolation

Sorbitol was measured by gas chromatography [8]. The sorbitol peak was confirmed by mass spectrometry. AR was immunoprecipitated from heart tissue using polyclonal anti-AR antibodies. Glutathiolation of AR was measured by Western analysis using anti-PSSG antibodies (Virogen, Cambridge, MA).

2.6. Data and statistical analysis

Data are presented as mean ± S.E.M. and the *P* values were determined using the unpaired student's *t*-test.

3. Results

3.1. L-Arginine increases NO production in hyperglycemic mice

Four weeks after STZ treatment, there was no change in heart weight or heart/body weight ratios in mice treated with saline or L-arginine (Table 1). Non-fasting blood glucose was significantly increased in STZ-treated mice. Although L-arginine has been reported to protect rat β-cells against the diabetogenic effects of alloxan [10], in our study, blood glucose levels were not affected by L-arginine treatment. High levels of NO_x were measurable in untreated mice; however, the levels of NO_x in the plasma of STZ-treated mice were undetectable. L-Arginine restored plasma NO_x production to a level not significantly different than non-diabetic animals (Table 1).

3.2. L-Arginine induces glutathiolation of AR and abolishes hyperglycemia-induced increases in sorbitol accumulation

Upon comparison with non-diabetic mice, a 5.8-fold increase in renal sorbitol level was observed in diabetic mice, indicating an increase in AR mediated reduction of glucose. No change in AR protein levels was observed (data not shown). Accumulation of sorbitol in diabetic animals was completely abolished by L-arginine (Fig. 1A), indicating that L-arginine inhibits the polyol pathway. L-Arginine treatment in diabetic animals also induced a marked increase in AR glutathiolation compared with diabetic hearts from mice treated with saline (Fig. 1B). The extent of AR glutathiolation induced by L-arginine was greater in STZ-treated animals than in control (non-diabetic) animals (Fig. 1B), indicating that AR in diabetic tissues is more susceptible to glutathiolation.

3.3. L-Arginine prevents PKC activation in the diabetic heart

Diabetic hearts displayed a 2.2-fold higher level of membrane-associated PKC activity than non-diabetic hearts

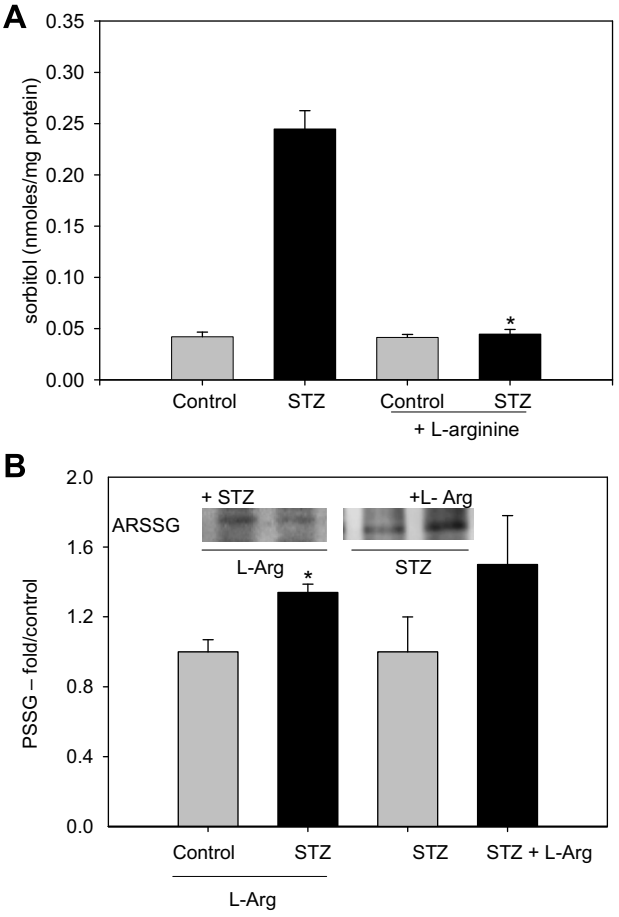

Fig. 1. L-Arginine prevents diabetic activation of AR and increases AR glutathiolation in the heart. (A) Sorbitol content was measured by gas chromatography in kidneys of non-diabetic and diabetic mice. Data are presented as mean ± S.E.M., $^*P < 0.001$ versus STZ, $n = 5–7$ per group. (B) AR glutathiolation was assessed by immunoprecipitation of AR from ventricular tissue followed by immunoblotting with anti-PSSG antibodies. Inset shows Western blots representative of three to four individual experiments. Data are presented as mean ± S.E.M., $^*P < 0.05$ versus control, $n = 3–4$ per group.

(Fig. 2A). There was significantly greater PKC-β_{II} phosphorylation in diabetic hearts than the non-diabetic controls. PKC-β_{II} activation in diabetic hearts was abolished by L-arginine treatment (Fig. 2B).

3.4. L-Arginine decreases vascular superoxide generation

STZ-induced diabetes was associated with a significant increase in dihydroethidium (DHE) staining of aorta. Compared with non-diabetic controls, a 6.2 ± 1.6-fold increase in fluores-

Table 1
Physical parameters of study animals

	Control	STZ	Control + L-arg	STZ + L-arg
Heart weight (g)	22.9 ± 0.43	21.1 ± 0.51	23.6 ± 0.55	17.8 ± 0.12
Heart/body ratio (mg/g)	4.96 ± 1.9	4.47 ± 1.4	4.63 ± 1.7	4.91 ± 2.8
Blood glucose (mg/dl)	258 ± 27	>400	278 ± 29	>400
NO_x (nitrate + nitrite) (micromol/l)	918 ± 386	ND	3030 ± 1910	368 ± 81.0

Blood glucose levels were measured in non-fasting animals. Plasma NO_x levels were measured by colorimetric reaction in plasma samples that were filtered to remove all proteins above 10 kDa. NO_x were not detectable (ND) in plasma from diabetic mice (STZ). Values are mean ± S.E.M., $n = 5–8$.

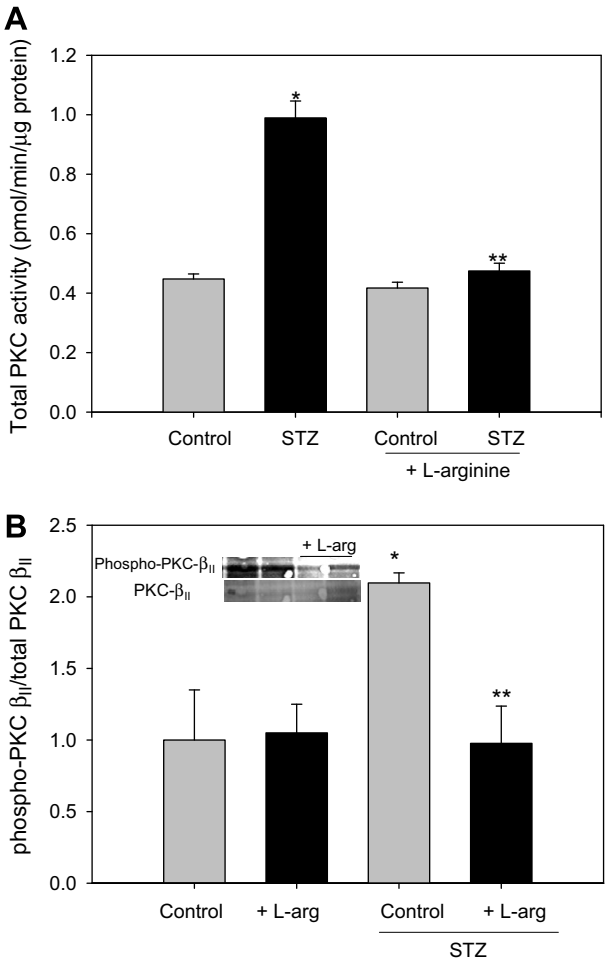

Fig. 2. L-Arginine supplementation abolishes diabetes induced PKC activation. (A) Membrane-bound total PKC activity in ventricular tissue from mouse hearts was measured and normalized to total protein. Data are presented as mean ± S.E.M., $^{*}P < 0.002$ versus control, $^{**}P < 0.002$ versus STZ, $n = 3$ per group. (B) PKC-β_{II} phosphorylation was measured by Western blot analysis in mouse hearts and normalized to total PKC-β_{II} in the same blot after stripping and reprobing. Data are presented as mean ± S.E.M., $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus STZ, $n = 3$–4 per group.

cence was observed (Fig. 3A and B). L-Arginine treatment significantly decreased DHE staining compared with saline-treated diabetic animals (Fig. 3B).

3.5. L-Arginine prevents diabetic dyslipidemia

Diabetic mice displayed 2- to 2.4-fold higher levels of plasma triglyceride than non-diabetic controls. L-Arginine treatment reduced plasma triglycerides in diabetic animals to levels not significantly different from those of non-diabetic animals (Fig. 4A). No differences in total cholesterol were observed (Fig. 4B). NMR analysis revealed that L-arginine treatment significantly decreased triglycerides associated with VLDL (40.9 ± 12.9 mg/dl versus 17.3 ± 14.6 mg/dl; $P < 0.05$), which was associated with a slight, but statistically insignificant decrease in VLDL particle concentration. No significant changes in VLDL particle size was observed, although the concentration of large VLDL was decreased from 3.07 ± 1.7 to 0.52 ± 0.3 nmol/l ($P < 0.05$).

3.6. L-Arginine diminishes vascular inflammation

Vascular inflammation, as assessed by an increase in the circulating levels of soluble intercellular adhesion molecule (sICAM), is increased in T1D [11]. In STZ-treated mice, we observed a modest but significant increase (1.3-fold versus control) in sICAM levels. This increase in sICAM was completely abolished by L-arginine (Fig. 5), suggesting that L-arginine prevents endothelial activation. L-Arginine treatment did not significantly change circulating TNF-α. Lower levels of plasma TNF-α were observed in diabetic animals treated with L-arginine than either non-diabetic or untreated diabetic animals, although the difference was not statistically significant (data not shown).

4. Discussion

The results of this study show that L-arginine treatment ameliorates vascular inflammation and diabetic dyslipidemia in a murine T1D model. Treatment with L-arginine markedly prevented tissue sorbitol accumulation, ROS generation, and PKC activation – three critical biochemical abnormalities associated with hyperglycemic injury. Taken together, these results support the novel concept that NO is an endogenous negative regulator of the AR–PKC–ROS pathway and therefore a decrease in NO production or availability during diabetes is a key step that is mechanistically linked to the major biochemical effects of high glucose. It follows that increasing NO synthesis by L-arginine could prevent oxidative stress and inflammation by restoring the regulatory axis of NO that controls glucose metabolism via AR and the downstream activation of PKC and ROS production.

Changes in NO signaling and production profoundly affect cardiovascular disease [12,13]. Indeed, a decrease in NO has been suggested to be the underlying mechanism common to all major CVD risk factors such as high cholesterol, hypertension, and smoking [12,13]. Diabetes is associated with profound impairment of NO production and signaling. The insulin-resistant state of T2D is associated with marked endothelial dysfunction, which has been variably linked to a decrease in NO synthesis or NO bioavailability [1,2]. Diabetes has also been associated with increased NOS uncoupling leading to superoxide generation [14]. It is likely that the role of NO in regulating CVD and cardiovascular complications of diabetes is complex and depends on disease stage and duration. Hence, for mechanistic understanding and therapeutic strategizing, it is essential to demarcate how the role of NO evolves and varies with disease progression and how disruptions in NO production and signaling can be restored.

Accordingly, in the current study, we examined the earliest stages of T1D, before the development of overt tissue injury (e.g., endothelial dysfunction), but when the biochemical changes are fully manifest. Targeting early stages of the disease is important, because during later stages of the disease, changes due to evolving systemic inflammation, insulin resistance and dyslipidemia become circuitous, making it difficult to separate causes from effects. In particular, the synergistic effect of glucotoxicity and lipotoxicity become difficult to disentangle. However, as shown by the current study, during early stages of the disease, the biochemical changes are mainly due to high glucose and largely correctable by increasing NO. Although specific mechanisms of early NO deficiency remain

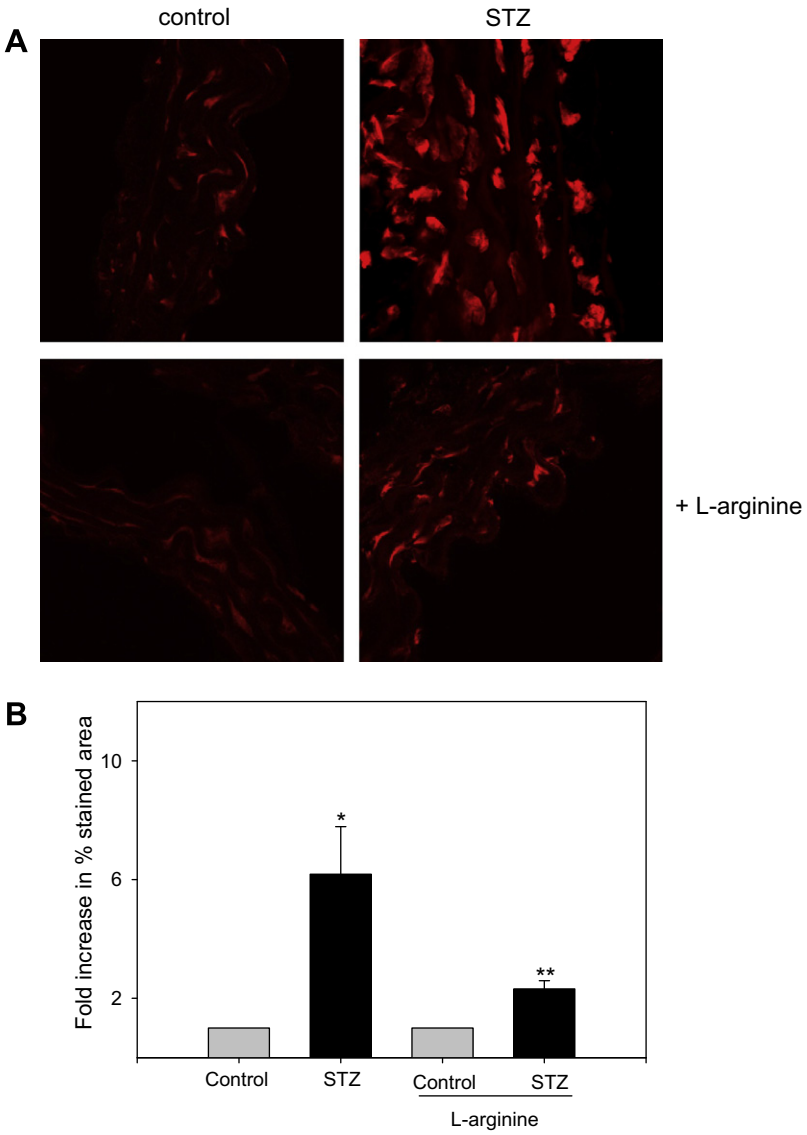

Fig. 3. L-Arginine supplementation decreases superoxide production in diabetic aorta. Sections (30 μm) of aorta from diabetic and non-diabetic mice were incubated with DHE to measure the abundance of superoxide. Panel A shows representative staining in aorta from control or diabetic animals (STZ) supplemented without or with L-arginine for 2 weeks (+L-arginine). (B) Fluorescent intensity was quantified using MetaMorph software. Plot represents fold-increase in thresholded area versus appropriate control. Data are presented as mean ± S.E.M., $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus STZ, $n = 5$–9 animals per group.

unclear (see below), the observation that biochemical changes due to high glucose were overcome by L-arginine treatment suggests that the decrease in NO bioavailability is not due to BH_4 deficiency. Moreover, the defect does not appear to be due to NOS uncoupling, because if this was so, then providing more substrate would have increased (rather than decreased) ROS production. Instead, it appears that the decrease in NO is due to its excessive utilization by increased ROS. This is consistent with the marked increase in superoxide generation in diabetic vessels, which depends upon the stimulation of several pathways including AR and PKC.

Previous studies with cells cultured in high glucose and STZ-treated rats show that the major pathways affected by high glucose are PKC [15] and AR [4]. High glucose and diabetes also lead to excessive accumulation of advanced glycosylation end products (AGEs) [3]. This, however, is a late phenomenon. In the current study we found that although sorbitol accumulation and PKC activity were increased in mice within 4 weeks of STZ treatment, no significant accumulation of AGEs was found in the heart (data not shown). In tissues in which glucose transport is not regulated by insulin (e.g., endothelium, nerve, kidney), glucose, at concentrations exceeding the glycolytic capacity, is metabolized via AR to sorbitol, which is further converted to fructose by sorbitol dehydrogenase [4]. The activity of the polyol pathway, in particular AR has been linked to several cell functions. It has been shown that inhibition of the enzyme prevents SMC growth, endothelial activation and adhesion, and inflammation resulting from the activation of NF-κB and AP-1 [4]. Significantly, inhibition of AR prevents high glucose-induced DAG formation and the activation of multiple PKC isoforms, including PKC-β_{II} [16]. Our previous work shows that NO inhibits AR activity by inducing the for-

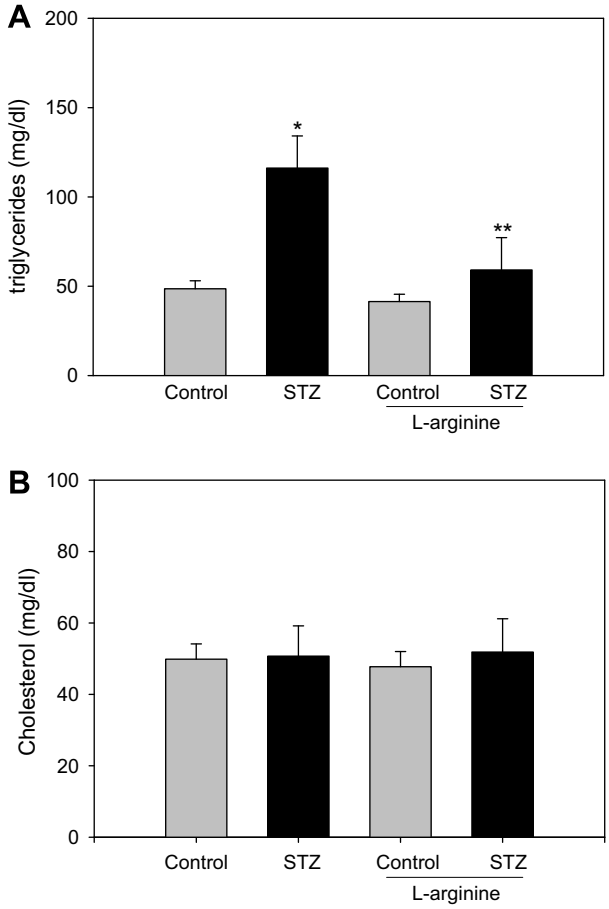

Fig. 4. L-Arginine supplementation diminishes triglycerides during diabetes. Plasma triglycerides (A) and total cholesterol (B) were measured as described. Data are presented as mean ± S.E.M., $^{*}P < 0.0005$ versus control; $^{**}P < 0.05$ versus STZ, $n = 6–9$.

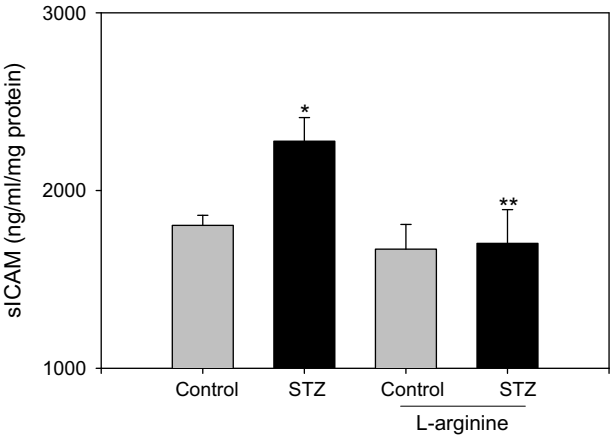

Fig. 5. L-Arginine treatment decreases circulating markers of inflammation. Plasma sICAM levels were measured by ELISA and normalized to total protein. Data are presented as mean ± S.E.M., $^{*}P < 0.05$ versus control; $^{**}P < 0.05$ versus STZ, $n = 5–7$ per group.

mation of a glutathione mixed-disulfide at the active site cysteine [4]. We have shown that treatment of diabetic rats with L-arginine or nitroglycerine inhibits AR activity and prevents sorbitol accumulation [8]. Consistent with this work, we found that L-arginine treatment prevents sorbitol accumulation in diabetic mice; however, the current data reveal for the first time that augmenting NO synthesis by L-arginine increases AR glutathiolation and prevents downstream increase in ROS generation and PKC activation.

Activation of PKC isoforms, particularly PKC-β_{II} has been associated with several features of glucose toxicity, including microvascular changes, increased vascular ROS generation, and endothelial and macrophage activation [17]. In particular, hyperglycemia-induced endothelial dysfunction in humans is prevented by pretreatment with the PKC-β_{II}-specific inhibitor – ruboxistaurin [18]. Hence, our observation that increasing NO by simple L-arginine supplementation prevents PKC activation supports the unifying hypothesis that AR plays a critical role in the early stages of diabetic complications and that inhibition of this enzyme by increasing NO prevents one of the major causes of diabetic complications, i.e., PKC activation.

We found that murine T1D was associated with an increase in plasma triglycerides. This is consistent with human data showing that poorly controlled T1D is associated with hypertriglyceridemia [19]. Treatment with L-arginine decreased total and VLDL-associated triglyceride content, consistent with a direct effect of NO on VLDL rather than on chylomicron remnants. Decreases in HDL cholesterol content and size were also observed (data not shown), which may be reflective of reversal of diabetes-induced changes. The decrease in VLDL triglycerides was; however, associated with a decrease in the concentration of large VLDL and an increase in IDL concentration. These changes most likely reflect a reduction in triglyceride concentration. Hence, the primary effect of L-arginine on lipoprotein subclasses reflects attenuation of hypertriglyceridemia. While the mechanisms by which L-arginine (or NO) affects triglyceride metabolism remain unclear, it is significant to point out that unlike L-arginine treatment, overexpression of GTP cyclohydrolase fails to normalize diabetes induced increases in triglycerides or HDL even though BH_4 levels were restored. Thus, L-arginine supplementation may be an effective intervention for treating early dyslipidemia in diabetes.

In summary, we have found that L-arginine supplementation prevents vascular oxidative stress and inflammation in a murine model of T1D diabetes. The results obtained support the hypothesis that NO is a master regulator of the AR–PKC–ROS pathway, which is the major mediator of hyperglycemic injury. Based on these observations, we suggest the L-arginine treatment (or other modes of increasing NO synthesis) may be a beneficial clinical intervention to suppress or delay hyperglycemic injury at least during the early stages of T1D.

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References

[1] Beckman, J.A., Creager, M.A. and Libby, P. (2002) Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. JAMA 287, 2570–2581.
[2] Creager, M.A., Luscher, T.F., Cosentino, F. and Beckman, J.A. (2003) Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy. Part I. Circulation 108, 1527–1532.
[3] Brownlee, M. (2001) Biochemistry and molecular cell biology of diabetic complications. Nature 414, 813–820.
[4] Srivastava, S.K., Ramana, K.V. and Bhatnagar, A. (2005) Role of aldose reductase and oxidative damage in diabetes and the

consequent potential for therapeutic options. Endocr. Rev. 26, 380–392.
[5] Lund, D.D., Faraci, F.M., Miller Jr., F.J. and Heistad, D.D. (2000) Gene transfer of endothelial nitric oxide synthase improves relaxation of carotid arteries from diabetic rabbits. Circulation 101, 1027–1033.
[6] Alp, N.J., Mussa, S., Khoo, J., Cai, S., Guzik, T., Jefferson, A., Goh, N., Rockett, K.A. and Channon, K.M. (2003) Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. J. Clin. Invest. 112, 725–735.
[7] Mendez, J.D. and Balderas, F. (2001) Regulation of hyperglycemia and dyslipidemia by exogenous L-arginine in diabetic rats. Biochimie 83, 453–458.
[8] Chandra, D., Jackson, E.B., Ramana, K.V., Kelley, R., Srivastava, S.K. and Bhatnagar, A. (2002) Nitric oxide prevents aldose reductase activation and sorbitol accumulation during diabetes. Diabetes 51, 3095–3101.
[9] Guzik, T.J., Mussa, S., Gastaldi, D., Sadowski, J., Ratnatunga, C., Pillai, R. and Channon, K.M. (2002) Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. Circulation 105, 1656–1662.
[10] Mendez, J.D. and Hernandez, R.H. (2005) L-Arginine and polyamine administration protect beta-cells against alloxan diabetogenic effect in Sprague–Dawley rats. Biomed. Pharmacother. 59, 283–289.
[11] Targher, G., Bertolini, L., Zoppini, G., Zenari, L. and Falezza, G. (2005) Increased plasma markers of inflammation and endothelial dysfunction and their association with microvascular complications in Type 1 diabetic patients without clinically manifest macroangiopathy. Diabet. Med. 22, 999–1004.
[12] Cooke, J.P. and Dzau, V.J. (1997) Nitric oxide synthase: role in the genesis of vascular disease. Annu. Rev. Med. 48, 489–509.
[13] Landmesser, U., Hornig, B. and Drexler, H. (2004) Endothelial function: a critical determinant in atherosclerosis? Circulation 109, II27–II33.
[14] Forstermann, U. and Munzel, T. (2006) Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 113, 1708–1714.
[15] Sheetz, M.J. and King, G.L. (2002) Molecular understanding of hyperglycemia's adverse effects for diabetic complications. JAMA 288, 2579–2588.
[16] Ramana, K.V., Friedrich, B., Tammali, R., West, M.B., Bhatnagar, A. and Srivastava, S.K. (2005) Requirement of aldose reductase for the hyperglycemic activation of protein kinase C and formation of diacylglycerol in vascular smooth muscle cells. Diabetes 54, 818–829.
[17] Rask-Madsen, C. and King, G.L. (2005) Proatherosclerotic mechanisms involving protein kinase C in diabetes and insulin resistance. Arterioscler. Thromb. Vasc. Biol. 25, 487–496.
[18] Beckman, J.A., Goldfine, A.B., Gordon, M.B., Garrett, L.A. and Creager, M.A. (2002) Inhibition of protein kinase Cbeta prevents impaired endothelium-dependent vasodilation caused by hyperglycemia in humans. Circ. Res. 90, 107–111.
[19] Ginsberg, H.N. (1996) Diabetic dyslipidemia: basic mechanisms underlying the common hypertriglyceridemia and low HDL cholesterol levels. Diabetes 45 (Suppl. 3), S27–S30.